Mono- and binuclear Zn-β-lactamase from *Bacteroides fragilis*: catalytic and structural roles of the zinc ions

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Abstract The *Bacteroides fragilis* Zn-β-lactamase is active with a mono- and a binuclear zinc site. The apoenzyme produced by removal of both Zn ions does not recover full activity upon readdition of Zn^{2+} in contrast to an active mono-Zn form prepared at pH 6.0. Differences in k_{cat} values observed are substrate-dependent implying distinct mechanisms for the mono- and binuclear species. The substrate profile of a Zn,Cd hybrid obtained by selective exchange of one zinc ion is different from that of the Zn_2 enzyme with a remarkable 15-fold increased activity with cefoxitin as substrate.

Key words: *Bacteroides fragilis*; Zinc β-lactamase; Zinc removal; Metal exchange; Thermal denaturation

1. Introduction

The Zn-β-lactamase produced by *Bacteroides fragilis* hydrolyses a broad spectrum of β-lactams including imipenem and cefoxitin [1–3]. The 3D structure, solved by X-ray crystallography [4,5], shows the presence of two Zn ions in the active site, both at pH 7.0 and at pH 9.0. Zn_1 is coordinated by the side-chains of His^{82}, His^{84} and His^{145} and Zn_2, 3.3–3.4 Å away from Zn_1, by those of Asp^{86}, His^{206} and Cys^{164} and a water molecule [6]. The Zn_2 form of the homologous *Bacillus cereus* enzyme shows a very similar Zn coordination [6]. In the *B. fragilis* enzyme a bridging hydroxide was observed between the two Zn ions, which was suggested to act as the nucleophile in the catalytic cleavage of the β-lactam bond [5]. So far the number of Zn ions essential for the activity of metallo-β-lactamases remains controversial. The *B. fragilis* enzyme exhibits a strong affinity for the two Zn ions and has been considered an obligatory binuclear enzyme [5]. Full catalytic activity vs nitrocefin is reached when two Zn ions are bound [7], but the activity of the mononuclear form cannot be deduced from these results since a cooperative binding of the two Zn ions is possible. The recently solved structure of an active Cd_2 form of the enzyme was found to be similar to that of the Zn_2 species [8], but in an inactive Hg,Zn hybrid, the mercury ion occupies a modified second site, 4.8 Å away from Zn_1, and is coordinated by Cys^{87} and Cys^{164}.

In the present work, selective removal of only one Zn ion allowed a comparison of the catalytic activities of the mononuclear and binuclear forms. One of the two Zn ions in the binuclear enzyme was also replaced by Cd^{2+} and the activity of the resulting Zn,Cd hybrid compared to that of the native Zn_2 enzyme.

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Abbreviations: IDA, iminodiacetic acid; AAS, atomic absorption spectroscopy; DTT, dithiothreitol; Nbs_2, 5,5′-dithio-bis(2-nitrobenzoate); DSC, differential scanning calorimetry

2. Materials and methods

The *B. fragilis* Zn-β-lactamase was purified as described by Carfi et al. [9]. Metal-depleted buffers with a residual level of approximately 20 nM of Zn^{2+} were obtained by stirring with 0.2–0.5% (v/v) of IDA agarose (Affiland, Liège, Belgium) [10]. When the experiments required metal-free conditions standard precautions were taken [11]. Unless otherwise stated, all experiments were performed in 25 mM HEPES buffer pH 7.5 containing 10 μM Zn^{2+}.

2.1. Metal content analysis

The enzyme cadmium and/or zinc content was measured with a Perkin Elmer 2100 atomic absorption spectrometer in the flame mode after dialysis of 0.35–0.5 ml samples of 50 μM enzyme against 100 ml of the specified buffers at 4°C. The protein concentration was determined spectrophotometrically using $\varepsilon_{279nm} = 34\,800\ M^{-1}\ cm^{-1}$.

2.2. Thermal denaturation

Thermal denaturation was studied (i) by monitoring the emission of fluorescence of a 1.5 μM enzyme solution at 340 nm (excitation wavelength: 280 nm) with a Perkin Elmer LS50 luminescence spectrometer and using a temperature gradient of 1°C/min from 25 to 85°C; (ii) in a Microcal Inc. MCS differential scanning calorimetry (DSC) unit after dialysis of the enzyme samples (40–65 μM) against the assay buffer in the absence or presence of 10 mM DTT. The dialysis buffers were used as references for the corresponding samples. The data were collected and analysed with a MCS Observer software package assuming a two-state (F ⇔ U) folding mechanism.

2.3. Inactivation by chelating agents and apoenzyme production

First-order inactivation rate constants were derived by determining residual activities with nitrocefin in metal-free buffer after increasing incubation periods of 0.5–1 μM enzyme at 4°C with the following chelating agents: ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2,6-pyridinedicarboxylic acid (dipicolinic acid) and 1,10-phenanthroline.

The apoenzyme was produced at 4°C by dialysis of 0.3–0.5 ml of 200 μM enzyme against 4–5 changes of 100 ml 25 mM HEPES pH 7.5 containing 2 mM dipicolinic acid. The chelator was removed by seven dialysis steps against the same buffer containing 0.1% of IDA agarose. Oxygen-free dialysis was performed after extensive flushing of buffer solutions with nitrogen. The resulting apoenzyme was handled in an argon atmosphere. All the apoenzymes produced showed less than 5% residual Zn^{2+} as judged by activity measurements in the absence of Zn^{2+} and by AAS.

2.4. Titration of -SH groups and kinetic measurements

The number of thiol groups of the protein was determined by monitoring the A_{412} value after addition of 1 mM Nbs_2 and 1% SDS to 5–10 μM enzyme samples at 25°C ($\Delta\varepsilon_{412} = 13\,600\ M^{-1}\ cm^{-1}$). An aver-

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age value of 2.73 ± 0.30 thiol groups per molecule was obtained for the binuclear Zn enzyme.

Cefoxitin and imipenem were from Merck Sharp and Dohme (Rahway, NJ, USA). Nitrocefin and benzylpenicillin were from Unipath (Oxford, UK) and Rhone Poulenc (Paris, France), respectively. The change in absorbance resulting from substrate hydrolysis was followed with a Perkin Elmer Lambda 2 UV/VIS spectrometer. The K_m and k_{cat} values were obtained by analysis of at least three complete hydrolysis time-courses [12] performed with different substrate and enzyme concentrations and at 25°C. The following Δε values were used: $\Delta\varepsilon_{482nm} = 15\,000\ M^{-1}\ cm^{-1}$ for nitrocefin, $\Delta\varepsilon_{235nm} = -775\ M^{-1}\ cm^{-1}$ for benzylpenicillin, $\Delta\varepsilon_{300nm} = -9000\ M^{-1}\ cm^{-1}$ for imipenem and $\Delta\varepsilon_{260nm} = -6600\ M^{-1}\ cm^{-1}$ for cefoxitin. Routinely, activity measurements were performed with 150 μM nitrocefin.

3. Results

3.1. Thermal denaturation

The *B. fragilis* enzyme contains three cysteine residues, none of which is involved in disulphide bonds, and DTT was added to preclude the formation of intra- and/or intermolecular disulphide bonds during unfolding. The unfolding transitions visualised by fluorescence in the absence and presence of 1 mM DTT are shown in Fig. 1 where the two curves were scaled to a value of 1000 for the initial fluorescence at 25°C. After cooling back to 25°C the recovery of activity was less than 10% when the denaturation/renaturation cycle was performed in the absence of DTT and nearly complete in its presence, showing that the phenomenon was reversible only in the latter case. Surprisingly, 2.6 ± 0.3 thiol groups were found in a 50 μM enzyme sample after thermal denaturation in the absence of DTT.

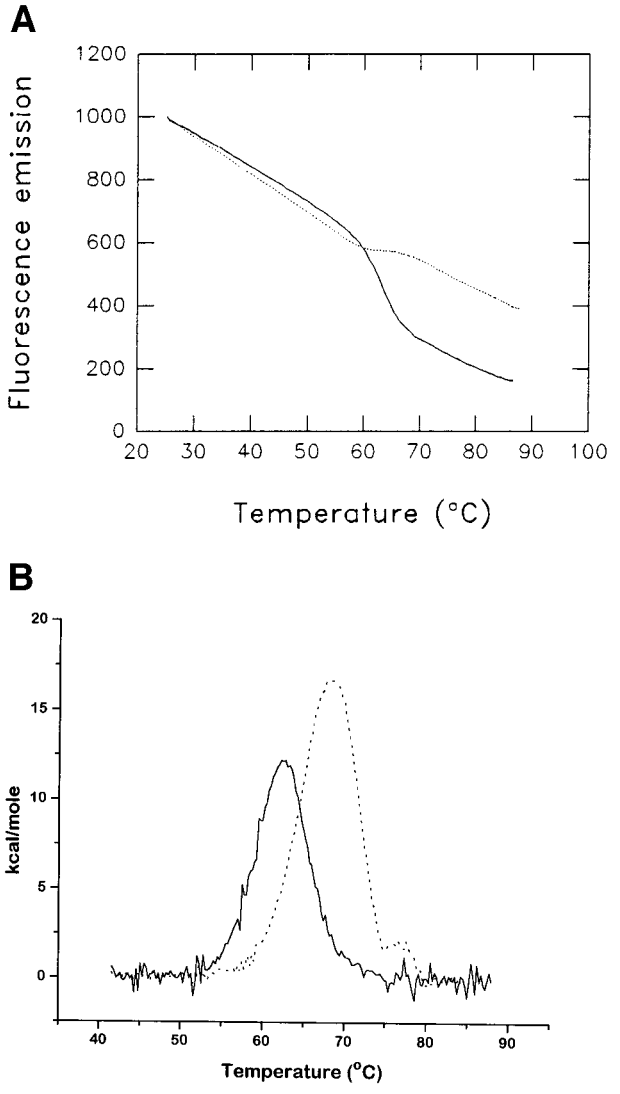

Fig. 1. Thermal denaturation monitored by fluorescence emission at 340 nm (A) and by DSC (B). The assay buffer was 25 mM HEPES pH 7.5 containing 10 μM Zn^{2+} in the absence of DTT (dashed lines) and in the presence of 1 mM (A) and 10 mM (B) DTT (solid lines).

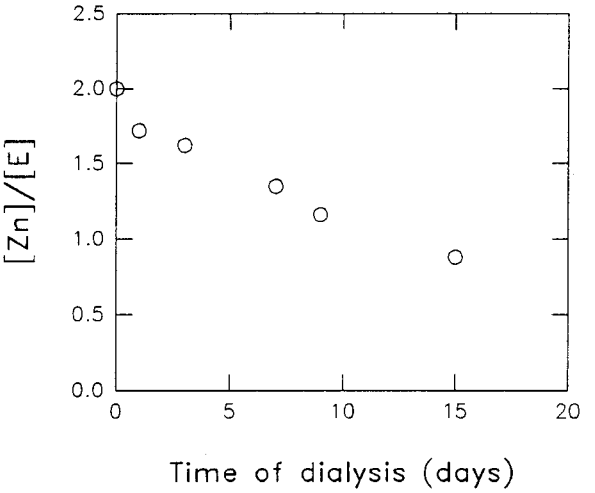

Fig. 2. Kinetics of Zn^{2+} release from the *B. fragilis* Zn_2-β-lactamase by dialysis against metal-free 25 mM Na-cacodylate pH 6.0.

Fig. 1B shows the DSC transitions of the thermal denaturation. For data representation, the enzyme concentrations (40.8 μM in the absence and 61.2 μM in the presence of 10 mM DTT) were normalised and the progress baseline subtracted. The reversible transition observed in the presence of 10 mM DTT could be fitted to a two-state model with T_m and H_m values of 62.2°C and 109 kcal/mol, respectively. By including the van't Hoff heat change (H_v) in the model, a H/H_v ratio of 1.2 was found indicating the presence of only one folding domain per enzyme molecule. In the absence of DTT and despite the irreversibility of the phenomenon, the data could again be fitted to a two-state model, with apparent T_m and H_m values of 67.9°C and 120 kcal/mol respectively.

Finally, hydrolysis of 150 μM nitrocefin was measured in the presence of DTT. The relative activities were 46% and 23% in 1 mM and 10 mM DTT, respectively.

3.2. Inactivation of the enzyme by chelating agents and apoenzyme production

The first-order inactivation rate constants were found to be linearly dependent on the chelating agent concentration (data not shown). Dipicolinic acid with a k_i of 0.11 min^{-1} at a concentration of 1 mM was the most efficient and was used to produce the apoenzyme as described above.

Apoenzymes produced under aerobic conditions recovered only 15–35% of activity in the presence of 10 μM Zn^{2+}. This result was not improved by preincubation of the apoenzyme in the presence of ≥ 10 μM Zn^{2+} for 1–24 h. Treatment with Nbs_2 revealed 50–70% free -SH groups per molecule with respect to the native enzyme. After dialysis of these samples

Table 1
Kinetic parameters of the *B. fragilis* enzyme with different $[Zn^{2+}]/[E]$ ratios in 25 mM HEPES pH 7.5

	Native Zn_2 enzyme			$[Zn^{2+}]/[E] = 1.2$			$[Zn^{2+}]/[E] = 1.9$		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Nitrocefin	4.6	260	57	4.4	210	48	–	250[a]	–
Benzylpenicillin	8.0	140	17	8.6	130	15	–	150[b]	–
Imipenem	140	210	1.5	160	190	1.2	140	200	1.4
Cefoxitin	120	14	0.12	120	32	0.27	100	12	0.12

The species exhibiting $[Zn^{2+}]/[E]$ ratios of 1.2 and 1.9 were obtained as explained in the text. k_{cat} values were derived from initial rates of hydrolysis of 150 μM nitrocefin and 1 mM benzylpenicillin. S.D. values are below 10%. The enzyme concentration was in the 0.005–0.15 μM range.

for 3 days against buffer containing 10 μM Zn^{2+} and 1 mM β-mercaptoethanol, the $[Zn^{2+}]/[E]$ ratio determined by AAS measurements was 2.2 ± 0.2. Despite the uptake of Zn^{2+} the activity recovery increased only by 5–10%.

The apoenzyme produced under anaerobic conditions contained 78% free -SH groups relative to the native Zn_2 enzyme and recovered 50% of activity upon addition of Zn^{2+}.

3.3. Correlation between Zn content and catalytic activity

AAS measurements on the enzyme samples after dialysis against 25 mM HEPES buffer pH 7.5 for 24 h always yielded 2.0 ± 0.2 Zn ions per enzyme molecule over external Zn^{2+} concentrations ranging from 1 to 200 μM.

The Zn^{2+} content of the *B. fragilis* enzyme after an 8-day dialysis against 25 mM HEPES pH 7.5, 25 mM HEPES pH 7.0, and 25 mM Na-cacodylate pH 6.5 metal-depleted buffers was 1.65 ± 0.10 equivalents of Zn per enzyme molecule. Dialysis of the enzyme against 25 mM Na-cacodylate pH 6.0 resulted in an accelerated release of Zn as shown in Fig. 2.

In order to investigate the activity of a Zn-depleted sample, 50 μM enzyme (1.5 ml) was dialyzed against 250 ml of 25 mM Na-cacodylate buffer pH 6.0. After 10 days of dialysis a $[Zn^{2+}]/[E]$ ratio of 1.2 was determined by AAS. Titration of thiolate groups on this sample with Nbs_2 revealed 96% of free -SH groups. In Table 1 the kinetic parameters for the samples with $[Zn^{2+}]/[E]$ ratios of 1.2 and 2.0 are compared for four different substrates.

The kinetic parameters of the native Zn_2 enzyme were identical in the absence (residual $[Zn^{2+}]$ about 0.35 μM) or presence of 10 μM Zn^{2+} in the assay buffer. This also applied to the sample exhibiting a $[Zn^{2+}]/[E]$ ratio of 1.2, indicating that no uptake of Zn^{2+} occurred during the activity measurements. Preincubation of the same sample in 25 mM HEPES buffer containing 10 μM Zn^{2+} for 1–24 h did not restore an activity similar to that of the binuclear enzyme. By contrast, after a 5-day dialysis against the same buffer the $[Zn^{2+}]/[E]$ ratio was 1.9 ± 0.2 and the kinetic parameters characteristic of the binuclear enzyme were restored (Table 1).

3.4. Production and catalytic activity of the mixed Zn,Cd species

Table 2 presents the Zn^{2+} and Cd^{2+} content of the enzyme after dialysis of the native Zn_2 enzyme against Zn^{2+}-depleted buffer containing different concentrations of Cd^{2+}. Dialysis against 5–50 μM Cd^{2+}-containing buffers for 1 day resulted in partial replacement of one of the two equivalents of Zn^{2+} by Cd^{2+}. Replacement of one equivalent could be achieved either by dialysis against 50 μM Cd^{2+} buffer for 1 day or by dialysis against lower concentrations of Cd^{2+} for a longer period. Replacement of the second equivalent of Zn^{2+} seems to be much slower. By increasing the dialysis time and/or the Cd^{2+} concentration to 100 or 200 μM, some additional Zn^{2+} was exchanged but the pure Cd_2 species could not be obtained.

The catalytic activities of the Zn,Cd hybrid are also presented in Table 2. With nitrocefin and benzylpenicillin as substrates it was less efficient than the Zn_2 enzyme but with imipenem and cefoxitin the activity was about 2- and 15-fold higher, respectively.

4. Discussion

Earlier studies reported that addition of Zn to the *B. fragilis* enzyme folded in the absence of Zn^{2+} did not yield an active

Table 2
Metal exchange by dialysis of the *B. fragilis* Zn_2 enzyme against 25 mM HEPES pH 7.5 containing different Cd^{2+} concentrations ($[Cd^{2+}]_{ext}$) and catalytic activity of the partially Cd-substituted enzyme species

$[Cd^{2+}]_{ext}$ (μM)	Dialysis time (days)	$[Zn^{2+}]/[E]$	$[Cd^{2+}]/[E]$	Specific activity (%)			
				Nitrocefin	Benzylpenicillin	Imipenem	Cefoxitin
–	–	2.0	–	100 (10)	100 (5.5)	100 (4.8)	100 (0.39)
5	1	1.4	0.4	83	80	132	750
10	1	1.2	0.7	72	48	201	1220
25	1	1.1	0.8	67	35	214	1300
50	1	0.9	1.1	73	31	227	1450
10	3	1.1	0.9				
50	3	0.9	1.3				
100	6	0.9	1.1				
200	6	0.80	1.3				

The specific activities (μmol s^{-1} mg^{-1}, given in parentheses) were determined as initial rates of hydrolysis of 150 μM nitrocefin, 1 mM benzylpenicillin, 200 μM imipenem and 225 μM cefoxitin prepared in metal-free 25 mM HEPES buffer pH 7.5 containing the corresponding $[Cd^{2+}]_{ext}$. S.D. values were below 10% in all cases. The final enzyme concentrations were 7–25 nM in all cases.

enzyme [7]. All our attempts to produce the *B. fragilis* apo-β-lactamase also failed in the sense that the resulting apoenzymes could not be fully reactivated by addition of Zn. By removal of the two Zn ions from the native Zn_2 enzyme in the presence of oxygen the number of free -SH groups of the apoenzyme was significantly decreased which might suggest the formation of an intramolecular disulphide bond between Cys^{164} and Cys^{84} whose sulfur atoms are 6 Å apart in the Zn_2 enzyme [8]. However, after dialysis in the presence of mercaptoethanol, the apoenzyme could bind two equivalents of Zn^{2+} but did not recover activity. This non-native conformation is reminiscent of the inactive hybrid Zn,Hg species where the Hg^{2+} ion was found bound to Cys^{164} and Cys^{84} in a modified second site [8]. Possibly, this distorted site might also host a zinc ion. Even when special precautions were taken to minimise thiol oxidation during the preparation of the apoenzyme, no more than 50% of the initial activity could be recovered upon readdition of Zn^{2+}, which indicates again that even the reduced apoenzyme is prone to irreversible adoption of a non-native conformation.

By contrast, release of one zinc ion from the Zn_2 enzyme is slow but reversible and the sulphydryl content of the Zn_1 species is close to that of the Zn_2 form. This indicates that the most tightly bound zinc ion plays an important structural role in addition to its catalytic function. Indeed, oxidation of the cysteine residues only occurs in its absence.

The thermal denaturation of the enzyme was reversible only in the presence of DTT. However, after thermal denaturation in the absence of reducing agent, no oxidation of the Cys was observed indicating that DTT did not act by precluding the formation of unwanted disulphide bonds. The observed inhibition of the enzyme activity by DTT indicates a mechanism involving a direct interaction with the enzyme, most likely by binding to the Zn ions in the active site. Binding of DTT could decrease the T_m value in the DSC transitions by facilitating Zn^{2+} release from the protein. During renaturation DTT could either form an early complex with the folding protein or regulate the delivery of Zn^{2+} thus preventing the formation of non-native conformations.

Only one equivalent of Zn^{2+} can be replaced by Cd^{2+} in a relatively rapid manner as shown by dialysis of the Zn_2 enzyme against Cd^{2+}-containing buffers (Table 2). The selective replacement of Zn^{2+} by Cd^{2+} at the site with Cys as a ligand could be favoured by the higher affinity of thiols for Cd^{2+} than for Zn^{2+} [13]. The discovery of different rates of metal exchange for the two sites supplies an excellent method to obtain hybrid metal species.

Partially Zn-depleted species were obtained by dialysis against 25 mM Na-cacodylate buffer pH 6.0. The kinetic results obtained for the different substrates can be consistently explained by a sequential release of Zn^{2+} resulting in the Zn_1 species at pH 6.0. The Zn_1 species is probably favoured at low pH by a decrease of the affinity for the second zinc ion but the long time of dialysis required suggests that its release is kinetically hindered. The substrate affinity was found to be identical for the mono- and binuclear species and only an effect on k_{cat} was observed for some substrates. With nitrocefin the activity of the binuclear enzyme was about 25% higher than that of the mononuclear form whereas both forms show identical activities with benzylpenicillin and imipenem. With cefoxitin the mononuclear enzyme shows a three-fold increase of activity when compared to the Zn_2 enzyme. Interestingly, replacement of one Zn^{2+} by Cd^{2+} in the binuclear enzyme results in a 15-fold increase of its catalytic efficiency on cefoxitin. For each substrate the activity was differently modified when compared to that of the Zn_2 enzyme (Table 2). Due to the close proximity of the two binding sites it is likely that in a binuclear centre both metal ions directly participate in the mechanism and that their characteristics affect the efficiency of catalysis.

Thus forming a binuclear Zn enzyme is not necessary for catalysis by the *B. fragilis* metallo-β-lactamase. The second metal ion is not only not needed for catalysis but may even lead to a less efficient mechanism with some substrates. Furthermore, it is the most tightly bound equivalent of Zn^{2+} that is crucial for the structural integrity of the active site. In this case there are no obvious reasons for the acquisition of a binuclear Zn centre by the *B. fragilis* Zn-β-lactamase. The strict conservation of the ligating residues at the second metal binding site in all the enzymes of the family may be explained if a translocation of the metal ion between the two metal sites is needed during catalysis. The simultaneous presence of two Zn metal ions in the *B. fragilis* enzyme could be a consequence of the high [Zn^{2+}] present during enzyme production [2,9]. One can wonder if the mononuclear form does not predominate in vivo, where the [Zn^{2+}] is expected to be significantly lower.

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References

[1] Livermore, D.M. (1993) ASM News 59, 129–135.
[2] Yang, Y., Rasmussen, B.A. and Bush, K. (1992) Antimicrob. Agents Chemother. 36, 1155–1157.
[3] Hedberg, M., Edlun, C., Lindqvist, L., Rylander, M. and Nord, C.E. (1992) J. Antimicrob. Chemother. 29, 105–113.
[4] Concha, N., Rasmussen, B.A., Bush, K. and Herzberg, O. (1996) Structure 4, 823–836.
[5] Carfi, A., Duée, E., Paul Soto, R., Galleni, M., Frère, J.-M. and Dideberg, O. (1998) Acta Crystallogr. D 54, 47–57.
[6] Carfi, A., Duée, E., Galleni, M., Frère, J.M. and Dideberg, O. (1998) Acta Crystallogr. D 54, 313–323.
[7] Crowder, M.W., Wang, Z., Franklin, S.L., Zovinka, E.P. and Benkovic, S.J. (1996) Biochemistry 35, 12126–12132.
[8] Concha, N., Rasmussen, B.A., Bush, K. and Herzberg, O. (1997) Protein Sci. 6, 2671–2676.
[9] Carfi, A., Paul Soto, R., Martin, L., Petillot, Y., Frère, J.-M. and Dideberg, O. (1997) Acta Crystallogr. D 53, 485–487.
[10] Hernandez Valladares, M., Felici, A., Weber, G., Adolph, H.W., Zeppezauer, M., Rossolini, G.M., Amicosante, G., Frère, J.-M. and Galleni, M. (1997) Biochemistry 36, 11534–11541.
[11] Auld, D.S. (1988) Methods Enzymol. 158, 1–50.
[12] De Meester, F., Boris, B., Reckinger, G., Bellefroid-Bourgignon, C., Frère, J.M. and Waley, S.G. (1987) Biochem. Pharmacol. 36, 2393–2403.
[13] Krizek, B.A., Merkle, D.L. and Berg, J.M. (1993) Inorg. Chem. 32, 937–940.